IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Tod R. SMEAL *et al.*

Title: PHOSPHOSPECIFIC PAK ANTIBODIES AND DIAGNOSTIC KITS

Appl. No.: 10/716,936

Filing Date: November 20, 2003

Examiner: Sean E. AEDER

Art Unit: 1642

Conf. No. 6791

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Joseph Piraino, declare that:

1. I am a United States citizen and have been employed at PFIZER GLOBAL RESEARCH & DEVELOPMENT ("PFIZER"), located at 10724 Science Center Drive, San Diego, California 92121, since 1999. I have been employed at PFIZER as a Senior Scientist since 2004, and am responsible for managing and leading PFIZER's pharmacology group for various oncology projects which include designing and implementing pre-clinical feasibility studies and developing *in vitro* and *in vivo* animal models to study various pharmacological attributes of disease. I have appended a copy of my resume to this Declaration under **Exhibit A**.

2. I graduated from Boston University (Massachusetts), in 1995 with a Master of Arts (Concentration in Microbiology); and from the University of Massachusetts in 1993 with a Bachelor of Science in Chemistry. Please see **Exhibit A**.

3. I have read U.S. Application Serial No. 10/716,936 entitled "*Phosphospecific Pak Antibodies And Diagnostic Kit*," publication no. U.S. 2005/0054017. I do not have any personal interest in the outcome of the prosecution of this application.

4. I am familiar with the protein known as p21-activated kinase 4, *i.e.*, "PAK4" and its phosphorylation properties. For instance, it is known that phosphorylation of the serine residue at position 474 of the PAK4 protein activates kinase activity, and that activated PAK4 induces anchorage-independent cell growth, which facilitates the uncontrollable proliferation of affected cells in cancerous tissues. For this reason, phosphorylated PAK4 is typically found to be overexpressed in human tumor cells and that it can be a potent oncogenic factor in the body.

5. In 2005-2006, I conducted experiments to determine the effect of various PAK4 inhibitors on the serine-474 phosphorylation state of PAK4 in tumor tissues. Please see paragraph 9 below for specific details about my immunohistochemical staining protocol. First, I investigated the effect of PAK4 inhibitors on PAK4 ser-474 phosphorylation in non-small cell lung cancer and colon cancer tumor sections from mice, as explained in more detail below. My findings confirm that there exist compositions that reduce PAK4 phosphorylation at serine-474 in different tumor samples. A reduction in PAK4 phosphorylation at serine-474 means there are fewer activated PAK4 proteins in the cancer tissue that would otherwise contribute to oncogenic cell proliferation.

6. I observed that the PAK4 small-molecule inhibitor, which bears our in-house code, PF-02372468, causes a decrease in basal level of phosphorylation of PAK4 at activation residue serine-474. I took tumor sections from animals that had non-small cell lung cancer xenografted tumors ("A549"), and then processed those sections for immunohistochemistry staining after 18 hours of subcutaneous continuous infusion via Alzet pump of 140mg/kg/day PF-02372468 or a "vehicle" control.

7. **Exhibit B** depicts the results of the immunohistochemical staining of PAK4 serine-474-specific phosphorylation after exposure to PF-02372468 in comparison to total PAK4 phosphorylation levels and in comparison to a "vehicle"-only tumor control. My results show that the extent of PAK4 serine-474 phosphorylation in an animal with non-small cell lung cancer is *lower* when PF-02372468 had been administered to the animal as compared to the control. Compare the magnified inset in the bottom right hand corner of the "pPAK4^{ser474}" staining photographs between the vehicle-treated tumor control and the inhibitor-treated tumor sample.

8. I also conducted additional experiments to determine the effect of other PAK4 inhibitors on phosphorylation patterns in animals with colon cancer tumors and in the skin of mice. Specifically, I administered the small-molecule inhibitors PF-03685453 and PF-03678971 to animals that had colon carcinoma xenografted tumors ("HCT116"), and also to the skin of mice. Again, I took tumor sections from animals bearing the HCT116 colon tumors and processed them for immunohistochemistry after 18 hours of subcutaneous continuous infusion of 140mg/kg/day PF-02372468 or the vehicle-only control via Alzet pump.

9. **Exhibit C** shows the results of the PF-03685453 and PF-03678971 studies in photographic and graph formats. My observations were similar to those recorded for the non-small cell lung cancer data, namely that these other inhibitors (PF-03685453 and PF-03678971), *also* caused a decrease in the basal level of phosphorylation of PAK4 at activation residue ser474 in both the tumor section and the skin section compared to the vehicle-only control.

10. Specifically, the graphical data in **Exhibit C** shows that about 70% of cells in the vehicle-only control in the tumor sample pool stained positive for PAK4 ser-474 phosphorylation. However, less that 50% of cells treated with the PF-03685453 and PF-03678971 inhibitors stained positive for PAK4 ser-474 phosphorylation. This indicates a reduction of about 20% in phosphorylation of ser-474 in animals that had been treated with the inhibitors. I recorded a similar reduction in ser-474 phosphorylation pattern in the skin samples – the control samples had about 50% positive staining for ser-474 phosphorylation, whilst the skin of mice that had been treated with the inhibitors had fewer cells with serine-474-phosphorylated PAK4 proteins (about 40% and 30% for PF-03685453 and PF-03678971, respectively).

11. The following is a detailed explanation of the protocol I followed to stain the inhibitor-treated tumor biopsy samples described in paragraphs 6-8. First, I formalin-fixed tumor biopsies and then embedded them in paraffin. For immunohistochemical staining, I deparaffinized the slides prior to conducting the following treatments: for pPAK4 and PAK4 epitopes, which are described in paragraph [0052] of U.S. 2005/0054017, antigen retrieval consisted of heating the slides at 121°C at a pressure of 15 pounds in 10 mM citrate buffer at pH 6.0 by microwave (15 min, 700 W), and then cooling at room temperature for 20 min. After antigen retrieval, I incubated all slides for 30 min with 0.3% H_2O_2 in PBS to block endogenous peroxidase activity and washed them with PBS. I then incubated the slides with the primary antibody, which is disclosed in the application U.S. 2005/0054017, diluted in PBS-1%BSA (bovine serum albumin) for 60 min at room temperature. Primary antibodies were rabbit polyclonals anti-pPAK4 and anti-"total" PAK4 (1:200). I then washed the slides again with PBS. I then performed a secondary step by incubating the slides for 30 minutes at room temperature with goat anti-rabbit antibody conjugated to peroxidase (1:100 diluted in PBS-1%BSA with 1% goat serum) (DAKO, Glostrup, Denmark). After washing with PBS, I incubated the slides for 15 minutes with aminoethylcarbazole mixed with 50 µL 30% H_2O_2, and then counterstained the slides with haematoxylin for 1 minute. I then washed the slides with tap water before covering them with Kaiser's glycerine gelatine prior to visualization under a microscope. All standard laboratory chemicals were purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

12. I conclude that these experiments demonstrate that therapeutic compositions, such as the PAK4 inhibitors described in the preceding paragraphs reduce PAK4 phosphorylation on serine-474 in tumorogenic samples. Furthermore, reduction in PAK4 corresponds to anti-tumor activity in preclinical mouse xenograft models.

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I hereby declare that all the statements made herein of my known knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1 July 2008
DATE

JOSEPH S. PIRAINO

Piraino Declaration

Exhibit A

JOSEPH S. PIRAINO

9535 Easter Way, Apt. #4
San Diego, California 92121
(858) 405-7778
Email: joseph.piraino@pfizer.com

PROFESSIONAL SUMMARY

Energetic and dependable contributor with highly developed scientific and technical expertise complemented by interpersonal, managerial and computer skills. Self-starter and team player with results oriented focus. Excel in project advancement and commitment to division goals. Capable in learning and implementing high-throughput techniques & applications. Seek challenging opportunities on scientific projects in a matrixed organization with ever increasing levels of responsibility where specialized training, strong scientific abilities, and proven experience will be fully utilized and foster career growth.

EDUCATION

Boston University - Boston, Massachusetts **1995**
Master of Arts - Concentration in Microbiology - G.P.A. 3.8

University of Massachusetts - Boston, Massachusetts **1993**
Bachelor of Science in Chemistry - G.P.A. 3.6

BIOPHARMACEUTICAL LABORATORY EXPERIENCE

Pfizer Global Research & Development (formerly Agouron Pharmaceuticals) **San Diego, California**
Senior Scientist **2004 - present**
Research Scientist I **2003 - 2004**
Sr. Associate Scientist II **1999 - 2003**

As pharmacology lead (functional manager) for two oncology projects in an early phases of pharmacological, pre-clinical feasibility studies, have the full responsibility for all project-specific pharmacology tasks and deliverables including designing, developing and refining *in vitro/in vivo* models and assays for both screening and characterization of compounds as well as the timely collection, review and reporting of project data.
Oversee and coordinate all preclinical aspects - both *in vitro* and *in vivo* - of pharmacology support of a lead-seeking oncology project with a sub team of two full time dedicated scientists and two part time scientists.
Oversaw all *in vitro* pharmacology support for a lead-seeking oncology project including proof of concept studies, prelead selection, mechanism of action studies, and *in vitro* model characterization.
Planned and implemented biomarker studies for development of a noninvasive clinical assay for measurement of drug efficacy in tissues, blood and urine for one clinical and one lead-seeking project.
Supervised, managed training details, monitored performance, and provided feedback on scientific development of one full time employee, three temporary Associate Scientists and four Student Assistants in the lab.
Published posters and co-authored on various abstracts as well as research reports supporting IND filings.
Organize and coordinate oral presentations of progress in project team & departmental meetings.
Participate in decision making related to divisional goals & timelines and overall improvement in processes.
Perform experimental design/analysis, perform process and assay validation, troubleshoot areas of technical difficulty, and optimize conditions of bioassays with adequate documentation to protect intellectual property.

Advanced Biotherapies Corporation **San Diego, California**
Associate Scientist **1999**

Provided technical expertise in maintenance and improvement of gene therapy methodology and design. Assured adherence to SOP outlined by FDA in preparation of material used in a clinical trial. Performed animal experiments to study model of gene therapy for lung carcinoma. Surveyed operating policies, systems and procedures; assisted in a variety of functional areas as needed.
- Established, transfected and gene modified primary cell cultures for use in a clinical trial.
- Assayed sterility and assured quality control of clinical material by ELISA and other tests.
- Prepared GLP cell banks of numerous human cell lines for use in second clinical trial.

JOSEPH S. PIRAINO

Children's Hospital of Boston - Newborn Medicine — **Boston, Massachusetts**
Massachusetts General Hospital - GI Unit — **Boston, Massachusetts**
Research Technician II — **1994-1998**

Demonstrated technical proficiency in critical laboratory assignments at the cutting-edge of the discipline. Recognized and performed troubleshooting of problem technical areas; developed insights into the characterization and isolation of non-cellular substances conferring innate immunity; and pursued effective processes for determination of pertinent genetic regulatory elements. With hiatus.

· At Children's Hospital, conducted basic research project determining role of compromised innate immunity in disease processes; planned and structured training for post-doctoral research fellow and summer students; and submitted all results in detailed reports.

· At Mass. General, performed research, characterizing family of antimicrobial peptides.

Universidade de Brasilia - Multidisciplinary Laboratory — **Brasília, Brazil**
Visiting Researcher — **1996-1997**

Pursued and managed own original research project to elucidate molecular basis for phenomenon of autoimmunity associated with chronic Chagas disease. Championed an active motivational learning environment. Demonstrated creative and flexible pedagogical approaches and positive reinforcement skills. Encouraged appreciation of individual talents, skills and potential.

· Maintained a cooperative relationship with faculty, laboratory staff and students.

· Established own guidelines and priorities; organized purchasing of required resources.

· Conducted seminars and lectures in Portuguese.

Dana Farber Cancer Institute - Immunology — **Boston, Massachusetts**
Research Assistant — **1993**

Operated 2 FACScan flow cytometers using samples of cellular membranes conjugated with FITC tagged antibodies or nuclear samples stained with PI. Assisted core facility with scheduling.

University of Massachusetts at Boston - Chemistry Department — **Dorchester, Massachusetts**
Student Research Assistant — **1992-1993**

Provided work in a variety of functional areas as needed. Assisted MS student in an original research project using organic synthesis to create novel, nitrogenous condensing reagents.

LABORATORY EXPERTISE

DNA & RNA Manipulation - Sequenase & Cycle Sequencing - Subcloning - Extraction of Genomic DNA from Organs and Cell Lines - Genomic Library Construction & Southern Blotting - STS & Inverse PCR - In Vitro Mutagenesis - Electroelution - Operation of Oligo-Nucleotide Synthesizer - Culturing & Use of Recombinationally Deficient Bacterial Strains - mRNA Isolation & Northern Blotting - Oligo dT & Random 9-mer Primed cDNA Library Construction and Normalization via Subtraction Based Screening - RT-PCR - Primer Extension Protocols - Assay validation

Protein Chemistry - SDS/PAGE - Acid/Urea PAGE - Western Blotting - Affinity & Ion Exchange Chromatography - Immunoprecipitation & Immunoblotting - ELISA - Dialysis - Sonication

Cell & Tissue Culture - Electroporation & Chemical Transfection - Karyotyping - GLP Cell banking - MTT & XTT Assaying - Soft Agar Clonogenic Assaying - Radiation and Colony Based Assaying

Histotechnology & Animal Experience - Immunohistochemistry & Non-Radioactive In Situ Hybridization - Operation of a Cryostat & Microtome - Breeding of Reduviid Bugs - Large & Small Animal Handling - Injection of Mice Intravenously & Subcutaneously - Rodent Anesthesia - Passaging of Tumors in Nude Mice - Harvesting of Ascites - Establishment of Solid Tumors

Analytical – HPLC, FPLC and LC/MS – Liquid and Solid Phase Extraction of Small Molecules

JOSEPH S. PIRAINO

PUBLICATIONS

Piraino, J.S., Murray, B., VanArsdale, T., Popoff, I., Guo, A., Kraynov, E. and Los, G. Selective small molecule PIN1 PPIase domain inhibitors cause cell cycle modulation and characteristic cytotoxicity in cancer cells. *Cancer Research manuscript in preparation*

Bloom, L.A., Piraino, J.S., Yang, X., Los, G., and Boritzki, T.B. Methylthioadenosine phosphorylase may provide a means to exploit synthetic lethality and increase the therapeutic index of the de novo purine synthesis inhibitor AG2037. *In preparation*

Bloom, L.A., Piraino, J.S., Lorenzana, E.G., and Boritzki, T.B. Synthetic lethality and cancer chemotherapy: Selective prevention of AG2037-induced toxicity through the methylthioadenosine phosphorylase (MTAP) purine salvage pathway. Oral presentation AACR 2004.

Higgins, J.R, Piraino, J.S., Kingtree, J., Troche, G.E., Yang, X., Weller, D.G., Los, G. and Bloom, L.A. Selective rescue from toxicity induced by the antifolate antimetabolite AG2037 by methylthioadenosine. Proc AACR 2004; 4624.

Piraino, J.S., Boritzki, T.J., Hunter, R., Acena, A.M., Timple, N., Lamond, A.J., Arp, K.A., and Bloom, L.A. AG2037 Causes Apoptosis and Necrosis Selectively in Transformed Cells of Lymphoid Origin by Prolonged Depletion of Purine Ribonucleotide Pools. Proc. AACR – Apoptosis and Cancer: Basic Mechanisms and Therapeutic Opportunities in the Post-Genomic Era. 2002; B-35.

Bloom, L.A., Piraino, J.S., Yang, X.H., and Bartlett, C.A. The Antifolate AG2037 Utilizes Hypoxanthine Salvage As an Important Rescue Mechanism. Proc AACR 2002; 295.

Lamond, A.J., Piraino, J.S., Yang, X.H. and Bloom, L.A. The effect of p53 status on the cellular actions of the antifolate GARFT inhibitor AG2037. Proc. AACR 2002; 296.

Nizet V., Ohtake T., Lauth X., Trowbridge J., Rudisill J., Dorschner R.A., Pestonjamasp V., Piraino J., Huttner K., Gallo R.L. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001 Nov 22; 414 (6862): 454-7

Piraino, J.S., Boritzki, T.J., Higgins, J.R., Hunter, R., Acena, A., Arp, K.A., and Bloom, L.A. Oral Bioavailability of AG2037 is Not Affected by Simultaneous Administration with Inosine But is Hindered by Simultaneous Administration with Folic Acid. Proc. ASCO 2001; 20.

Piraino, J.S., Boritzki, T.J., Zhang, C.A., Stempniak, M.M., and Bloom, L.A. Examination of the Mechanism of Action of AG2037 by Measurement of *In Vitro* and *In Vivo* Purine Nucleotide Pools in Normal and Tumor Cells. Proc. AACR 2001, 42: 511.

Greig, M., Milgram, E., Zhang, C., Yan, Z., Piraino, J. and Boritzki, T.J. Biomarker LC/MS Method Development at PGRD La Jolla. Symposia 2001, Paris, France.

Bloom, L.A., Bartlett, C.A., Piraino, J.S., Margosiak, S., Dagostino, E.F., Neuffer, H.B., Arp, K.A. and Boritzki, T.J. Biochemical and Pharmacological Properties of AG2037, an Antifolate Inhibitor of De Novo Purine Synthesis. *In preparation*

Huttner, K.M., Piraino, J., and Gallo, R.L. Mus musculus cathelin-like protein and Mus musculus cathelicidin (Cramp) gene Genbank Accessions NM_009921, Nov. 2000 & AF035680, Dec. 1997.

Arganaraz, E.R., Barros, A.M., Simoes-Barbosa, A.M., Piraino, J.S. and Teixeira, A.R.L. Mus musculus clone A transfected with Trypanosoma cruzi kinetoplast sequence. Genbank Accessions AF002199, AF002200, AF002201, AF002202, AF002203. March 1999.

SPECIALIZED TRAINING

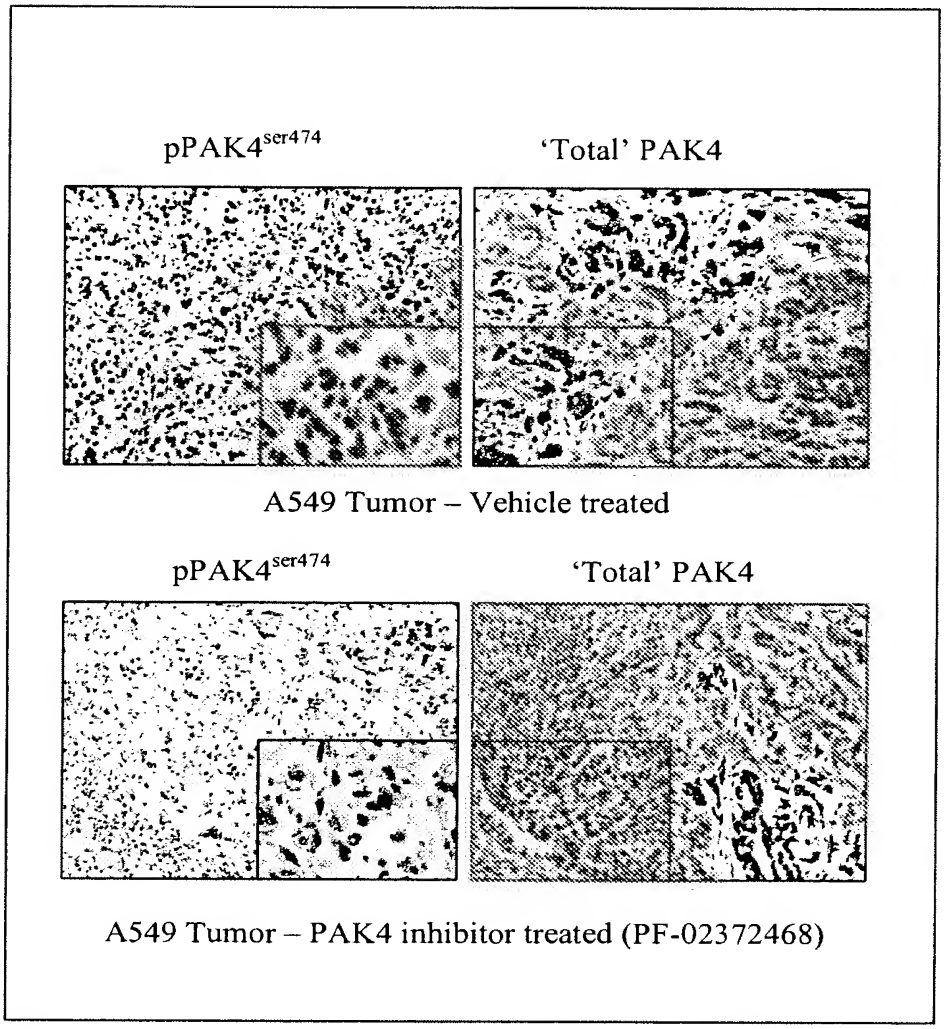
University of Wisconsin – Madison, Wisconsin **2001**
Basic Pharmacology short course - Diploma

Harvard University Medical School – Boston, Massachusetts **2000**
Cancer Medicine and Hematology short course – Diploma

Piraino Declaration

Exhibit B

EXHIBIT B

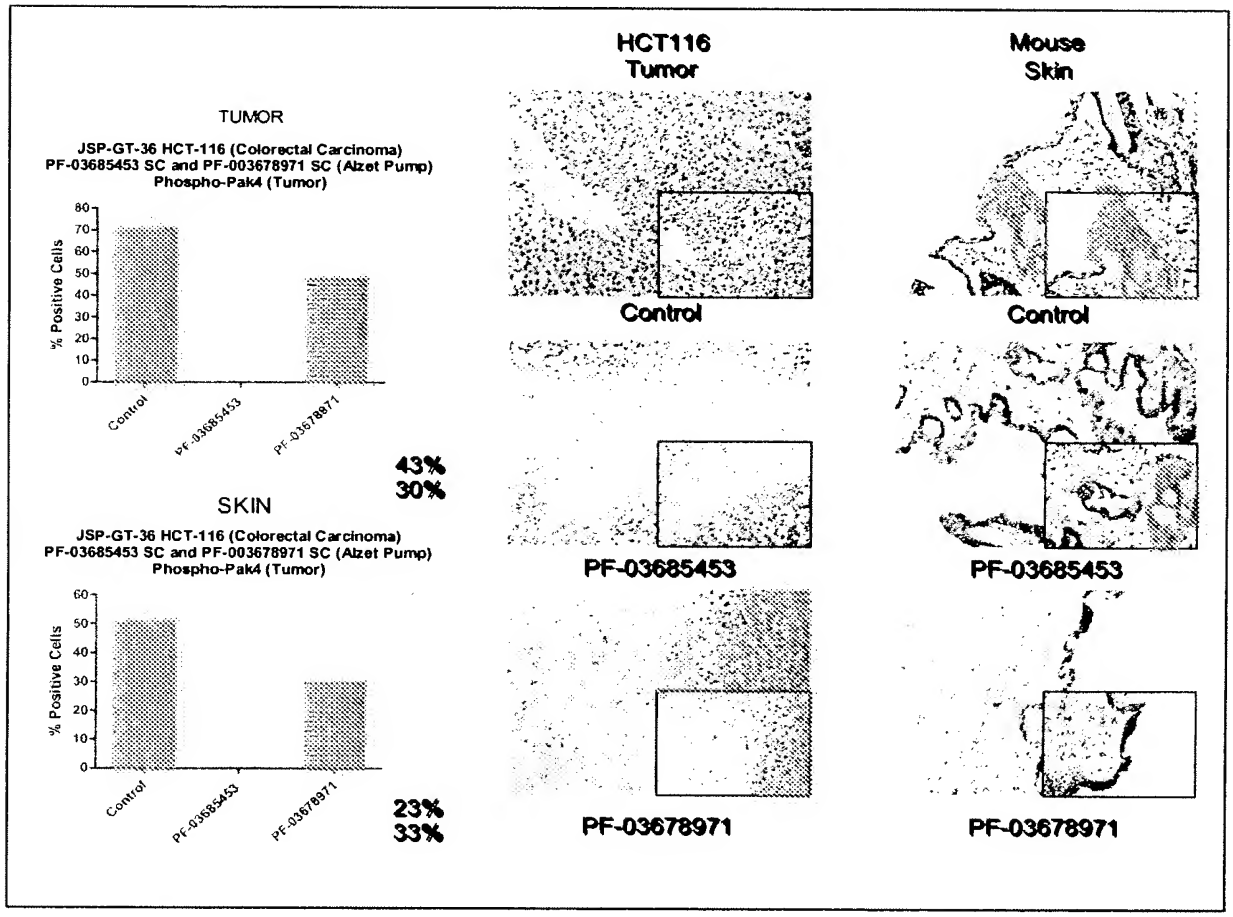


PAK4 inhibitor, PF-02372468, causes a decrease in basal level of phosphorylation of PAK4 at activation residue ser^{474}. Tumor sections were taken from animals bearing A549 NSCLC xenografted tumors and processed for immunohistochemistry after 18 hours of subcutaneous continuous infusion of 140mg/kg/day PF-02372468 (or vehicle) via Alzet pump.

Piraino Declaration

Exhibit C

EXHIBIT C



PAK4 inhibitors, PF-03685453 & PF-03678971, cause a decrease in basal level of phosphorylation of PAK4 at activation residue ser^{474} in tumor & skin. Tumor sections were taken from animals bearing HCT116 colon carcinoma xenografted tumors and processed for immunohistochemistry after 18 hours of subcutaneous continuous infusion of 140mg/kg/day PF-02372468 (or vehicle) via Alzet pump.